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The selective neurokinin 1 receptor antagonist R116301 modulates photic responses of the hamster circadian system

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Abstract

The recent development of selective NK_1 receptor antagonists that are active in vivo provides an important research tool to examine the role of substance P in the regulation of circadian rhythmicity. First, we tested whether R116301 {(2R-*trans*)-4-[1-[3,5-bis(trifluoromethyl)benzoyl]-2-(phenylmethyl)-4-piperidinyl]-*N*-(2,6-dimethylphenyl)-1-acetamide (S) hydroxybutanedioate}, a new selective NK_1 antagonist, alters the phase-shifting effects of light. Hamsters housed in constant darkness were injected with different doses of R116301, just before being exposed to a light pulse during the subjective night. The results were compared with those obtained with the NK_1 antagonist L-760,735 {2-(R)-(1-(R)-3,5-bis(trifluoromethyl)phenyl)ethoxy)-4-(5-(dimethylaminomethyl)-1,2,3-trioazol-4-yl)methyl-3-(5)-phenyl)morpholine}. Second, the effects of the NK_1 antagonists R116301 or L-760,735 injected immediately after exposure to a light pulse were similarly determined. Third, we investigated whether R116301 or L-760,735 injected during the mid-subjective day or the late subjective night can phase-shift the circadian rhythm of locomotor activity in hamsters housed in constant light. Both compounds reduced, by more than 30%, the phase-advancing effects of a light pulse in hamsters otherwise maintained in constant darkness, only when the drugs were administered before the light pulse. Under constant light conditions, both NK_1 receptor antagonists induced significant phase-advances when injected during the subjective day, but not during the subjective night. The present results indicate that tachykinergic neurotransmission modulates the photic responses of the circadian system upstream of phase resetting mechanisms and suggest that an inhibition of the NK_1 receptor signals "darkness" to the circadian clock.

Keywords: Substance P; Tachykinin; NK_1 receptor; Circadian rhythm; Suprachiasmatic nucleus

1. Introduction

The main circadian clock that regulates most biochemical, physiological and behavioral rhythms in mammals is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Klein et al., 1991; Miller et al., 1996). In the absence of external temporal signals, this circadian clock is capable of generating circadian signals that control near 24-h rhythms throughout the organism, although under natural conditions the light–dark cycle synchronizes the SCN clock to the exact 24-h period of the rotation of the earth on its axis. Light cues for photic synchronization are relayed directly from the retina to the SCN via the retino-hypothalamic tract, as well as indirectly from the intergeniculate leaflet (IGL) of the thalamus which sends a direct projection to the SCN (Morin, 1994; Miller et al., 1996).

A number of reports suggest that substance P plays a role in the overall circadian organization in mammals. Substance P is found in amacrine and ganglion cells of the retina (Brecha et al., 1987; Li et al., 1999), the retino-hypothalamic axons projecting to the SCN (Takatsuji et al., 1995; but see also Hartwich et al., 1994), the SCN (Mikkelsen and Larsen, 1993; Otori et al., 1993; Hartwich et al., 1994) and the IGL (Hartwich et al., 1994; Moore and Card, 1994). Treatment with substance P in in vitro preparations can phase shift the circadian rhythm

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of neuronal firing activity in the SCN at circadian times when light pulses induce phase shifts in the locomotor activity rhythm of animals housed in constant darkness (Shibata et al., 1992). Substance P also stimulates the firing rate and glucose uptake in SCN neurons (Shibata et al., 1992; Shirakawa and Moore, 1994; Piggins et al., 1995). Microinjections of substance P induce small phase-delays in the hamster activity rhythm only when delivered during the early subjective night (Piggins and Rusak, 1997). Local injections of spantide, a non-specific substance P receptor antagonist, reduce the light-induced expression of Fos in the SCN (Abe et al., 1996). These observations have given rise to the hypothesis that substance P participates in the entraining effects of light on the circadian system (Shirakawa and Moore, 1994).

The tachykinin family of neuropeptides, including substance P and neurokinins (NK) A and B, bind to three specific receptors, designated as NK_1, NK_2 and NK_3. The NK_1 receptor subtype is notably expressed in the circadian timing system, including the retina, the SCN and the IGL (Mick et al., 1995; Takatsuji et al., 1995; Casini et al., 1997). This receptor subtype is thought to mediate the modulation of substance P in the photic regulation of the circadian timing system (Shirakawa and Moore, 1994; Takatsuji et al., 1995; Challet et al., 1998). Our previous data indicate that the specific NK_1 receptor antagonist, L-760,735 {2-(R)-(1-(R)-3,5-bis(tri- fluoromethyl) phenyl)ethoxy)-4-(5-(dimethylamino- methyl)-1,2,3-trioazol-4-yl)methyl-3-(5)-phenyl)morpho- line}, is able to induce phase-advances in the circadian rhythms of hamsters kept in constant light. Furthermore, phase-advances of the locomotor activity rhythm that are normally induced by a light pulse during the late subjective night are reduced by pre-treatment with L-760,735, while light-induced phase-delays are unaffected (Challet et al., 1998). In order to further investigate the role of the NK_1 receptor in the regulation of the circadian clock system, we examined the effects of R116301 {(2R-*trans*)-4-[1-[3,5-bis(trifluoromethyl)benzoyl]-2(phenyl-methyl)-4-piperidinyl]-N-(2,6dimethylphenyl)-1-acetamide (S) hydroxybutanedioate}, a new tachykinin NK_1 antagonist (Romerio et al., 1999), on circadian rhythmicity in hamsters maintained under different lighting conditions. The results were compared with those obtained with L-760,735.

2. Materials and methods

2.1. Animals and recording of locomotor activity

Male Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles–River Lak:LVG (Saint-Aubin-les-Elbeuf, France) and remained under a 14 h light–10 h dark cycle for 2 weeks prior to the start of each experiment. During daytime, light intensity was 90–100 lux at the level of the cages. All animals were individually housed with access to a running wheel (diameter: 17 cm) for the continuous recording of wheel-running activity using the Chronobiology Kit (Stanford Software Systems, Stanford, CA).

2.2. Drugs and reagents

R116301 {(2R-*trans*)-4-[1-[3,5-bis(trifluoromethyl)-benzoyl]-2-(phenylmethyl)-4-piperidinyl]-N-(2,6-dimethylphenyl)-1-acetamide (S) hydroxybutanedioate} and L-760,735 {2-(R)-(1-(R)-3,5-bis(trifluoromethyl)phenyl)-ethoxy)-4-(5-(dimethylaminomethyl)-1,2,3-trioazol-4-yl)methyl-3-(5)-phenyl)morpholine} were synthesized in the laboratories of Janssen Research Foundation. These compounds bind selectively to the NK_1 receptor subtype in mammals and can cross the blood–brain barrier after peripheral injections (Kramer et al., 1998; Romerio et al., 1999).

Both compounds were dissolved in saline with 20% cyclodextrin.

2.3. Experimental design

Experiment 1 was designed to investigate the reducing effects of the NK_1 antagonist R116301 on light-induced phase-shifts in the rhythm of locomotor activity in two separate studies. (1) In the first study, a dose–response curve was generated for the possible effects of the injection of R116301 on light-induced phase-advances. Twenty-four hamsters were housed in constant darkness. After at least 10 days, half the animals received a single i.p. injection of 1.25, 2.5, 5.0 or 10 mg/kg of R116301 in 0.5 ml of vehicle. The other half received 0.5 ml of vehicle. Thirty minutes after the treatment, hamsters were exposed to a light pulse at circadian time (CT) 19 (i.e., 7 h after the time of activity onset, designated as CT12), a time when light produces large phase-advances. For light stimulation, hamsters were individually exposed to 100 lux of fluorescent white light for 10 min. The experiment was then repeated after 10 days, using a cross-over design in which the animals received the alternate treatment. (2) According to the results of the first study, we studied the effects of a single i.p. injection of R116301 (5 mg/kg) on the phase-advancing effects of a light pulse. The results were compared with those obtained with L-760,735 (5 mg/kg). In addition, the effects of a single i.p. injection of R116301 (5 mg/kg) or L-760,35 (5 mg/kg) on the phase-delaying effects of a light pulse were determined. For that purpose, 16 hamsters were housed in constant darkness. A first group of eight animals was injected with vehicle or each of the NK_1 antagonists. Thirty minutes after the treatment, hamsters were exposed to a light pulse at CT19. The experiment was performed on three different occasions separated by 10 days, the animals receiving

each time one of the alternate treatments. A second group of eight hamsters was injected with R116301 (5 mg/kg), L-760,735 (5 mg/kg) or vehicle 30 min before being exposed to a light pulse at CT14, a time when light produces large phase-delays in the circadian activity rhythm of hamsters. As described above, animals were injected on three different occasions and they were allowed to free-run for 10 days between each injection. No animal received the same treatment more than once. For a given animal, the order of the three injections was determined randomly.

In experiment 2, we investigated the effects of a single i.p. injection of R116301 (5 mg/kg) or L-760,735 (5 mg/kg) on the phase-advancing effects of light pulses when drugs were administered after animals were exposed to a 10-min light pulse at CT19. This treatment was done on three occasions in six animals kept in constant darkness who were randomly injected with vehicle or each of the NK_1 antagonists. Animals were allowed to free-run for 10 days between each injection. No animal received the same treatment more than once.

Experiment 3 was designed to test the hypothesis that injections of the NK_1 antagonists may mimic the effects of dark pulses in animals kept in constant light. Dark pulses typically induce phase-advances and phase-delays in the circadian rhythm of locomotor activity when applied, respectively, during the mid-subjective day and the late subjective night (Boulos and Rusak, 1982; Ellis et al., 1982; Van Reeth and Turek, 1989). Therefore, 16 hamsters were kept in constant light (100 lux). A first group of eight animals was injected on three different occasions separated by 10 days with vehicle or each of the NK_1 antagonists at CT8. A second group of eight hamsters was similarly treated with injections occurring at CT19. No animal received the same treatment more than once. The order of injections was determined randomly.

2.4. Data analysis

To determine phase-shifts in the circadian rhythm of locomotor activity, regression curves were fitted by eye to the onsets of locomotor activity for the last 8–10 days before the treatment and projected to the day of the treatment. Regression lines fitted to the onsets of activity during the 8–10 days following the treatment were retroprojected to the day of the treatment. The magnitude of the phase-shift was calculated as the difference between these two lines.

The circadian period was assessed by the χ^2 periodogram (Chronobiology Kit software) over the 10 days before and after the treatment.

2.5. Statistical analysis

Values are means±SEM. Analyses of variance (ANOVA) with repeated measures followed by a Student–Newman–Keuls test were performed using SigmaStat software (Jandel Scientific, San Rafael, CA). For all experiments, there was no effect of the order of injections ($P>0.05$). This factor, therefore, was not taken into account for the final analysis.

3. Results

3.1. Experiment 1: injections of R116301 or L-760,735 before a light pulse in hamsters kept in constant darkness

When hamsters were treated with R116301 or L-760,735 before being exposed to a light pulse at CT19, the subsequent light-induced phase-advances were significantly reduced (Figs. 1 and 2). There was a significant effect of the dose of R116301 ($F_{3,20}=4.41$, $P<0.05$; Fig. 1). The magnitude of light-induced phase-advances was not affected by injections of 1.25 mg/kg of R116301 compared to those of vehicle (82±5 vs 91±6 min, $P>0.05$). In contrast, injections of 2.5, 5 or 10 mg/kg of R116301 significantly reduced light-induced phase-advances in comparison with alternate vehicle treatment (60±6 vs 87±7, 68±7 vs 109±3 and 54±9 vs 85±11 min, respectively, $P<0.05$; Fig. 1).

Both R116301 (5 mg/kg) and L-760,735 (5 mg/kg) reduced the light-induced phase-advances by 36 and 32%, respectively ($F_{2,23}=6.48$, $P<0.01$; Figs. 2 and 3). A light pulse applied at CT14 resulted in phase-delays

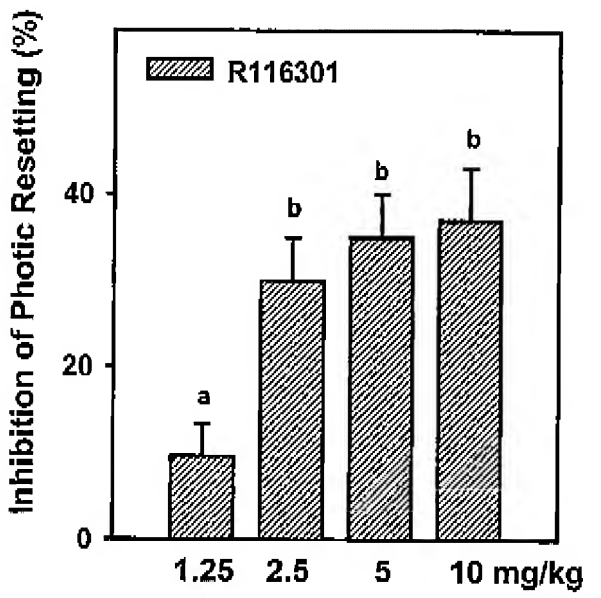

Fig. 1. Dose-dependence of R116301-induced reduction of photic phase advances. Each animal was kept in constant darkness and treated with vehicle or R116301 30 min before being to a light pulse (100 lux for 10 min) at CT19. Data are means±SEM ($n=6$ per group). Groups with no letters in common differ significantly from one another ($P<0.05$).

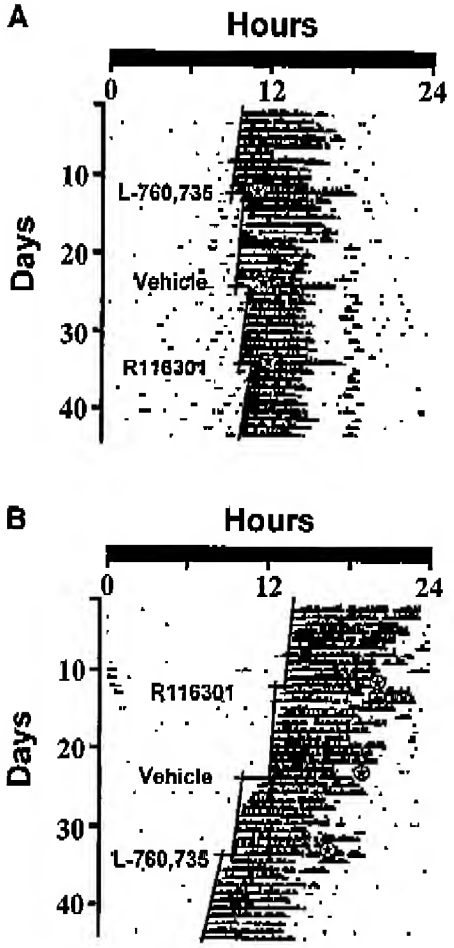

Fig. 2. Wheel-running activity of two hamsters housed in constant darkness. (A) This animal was exposed every 10 days to a light pulse (100 lux for 10 min) at CT14. Thirty minutes before the three light pulses, this animal was injected respectively with L-760,735 (5 mg/kg), vehicle, or R116301 (5 mg/kg). Time of injection is indicated by a star. (B) This animal was exposed every 10 days to a light pulse (100 lux for 10 min) at CT19. Thirty minutes before the three light pulses, this animal was injected respectively with R116301 (5 mg/kg), vehicle, or L-760,735 (5 mg/kg). Time of injection is indicated by a star.

of the free-running rhythm of locomotor activity (Fig. 2). The magnitude of the light-induced phase-delays was not affected by injections of the NK_1 antagonists R116301 and L-760,735 compared to those of vehicle (−54.4±4.6, −48.9±5.5 vs −45.4±4.6 min, respectively; $P>0.05$; Figs. 2 and 3). There were no significant changes in the circadian period before vs after the treatment at CT14 or CT19 ($F_{2,28}=0.38$, $P>0.1$; see Fig. 2 for examples).

3.2. Experiment 2: injections of R116301 or L-760,735 after a light pulse in hamsters kept in constant darkness

Light-induced phase-advances were not significantly altered when hamsters were treated with the NK_1 antagonists R116301 (5 mg/kg) or L-760,735 (5 mg/kg) after being exposed to a light pulse at CT19 in comparison with vehicle treatment (104.2±14.4, 106.3±14.9 vs 114.7±14.3 min, respectively; $F_{2,10}=0.48$, $P>0.05$; Fig. 4). The circadian period was not significantly affected by the treatment ($F_{2,10}=2.50$, $P>0.1$; data not shown).

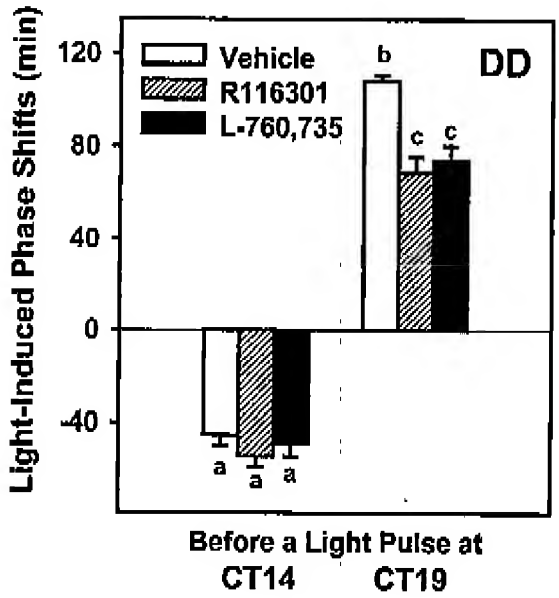

Fig. 3. Light-induced phase-shifts in circadian activity rhythms of hamsters pre-treated with the NK_1 antagonists, R116301 and L-760,735. Positive and negative values are advances and delays, respectively. Values are means±SEM ($n=7$ per group). Groups with no letters in common differ significantly from one another ($P<0.05$).

3.3. Experiment 3: injections of R116301 or L-760,735 in hamsters kept in constant light

Injections of R116301 (5 mg/kg) or L-760,735 (5 mg/kg) at CT8 led to phase-advances in the circadian

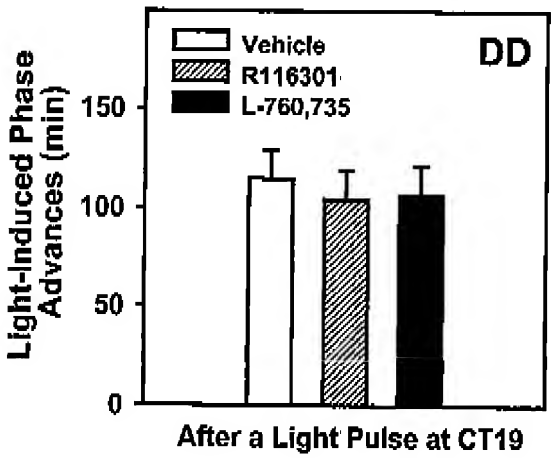

Fig. 4. Light-induced phase-advances in circadian activity rhythms of hamsters treated with the NK_1 antagonists, R116301 and L-760,735, after exposure to light. Values are means±SEM ($n=6$ per group). No significant difference was detectable between the groups ($P>0.05$).

tions (39.5±7.5, 62.0±22.0 vs 9.5±3.6 min, respectively; $F_{2,28}$=3.73, P<0.05; Figs. 5 and 6). The magnitude of the phase-advances was not different after injections of R116301 compared to that after injections of L-760,735 (P>0.05). Injections of R116301 (5 mg/kg), L-760,735 (5 mg/kg), or vehicle at CT19 had no phase-shifting effects (7.5±5.2, 2.4±6.5 and 1.6±3.9 min, respectively; Figs. 5 and 6). Because the increase in wheel-running activity during the subjective day can alter the circadian phase of locomotor activity in hamsters housed in constant light, we determined whether injections at CT8 trigger an increase in locomotor activity. The average number of wheel revolutions performed between CT8 and CT11 (i.e., during the 3-h period subsequent to the injections) was not significantly modified after the injections of R116301, L-760,735 or vehicle at CT8 (93±79, 240±136 and 160±107 wheel revolutions, respectively; $F_{2,14}$=0.58, P>0.1; see Fig. 5 for examples). The circadian period was not significantly affected by the injections of the NK_1 antagonists or vehicle at CT8 or CT19 ($F_{2,28}$=2.20, P>0.1; see Fig. 5 for examples).

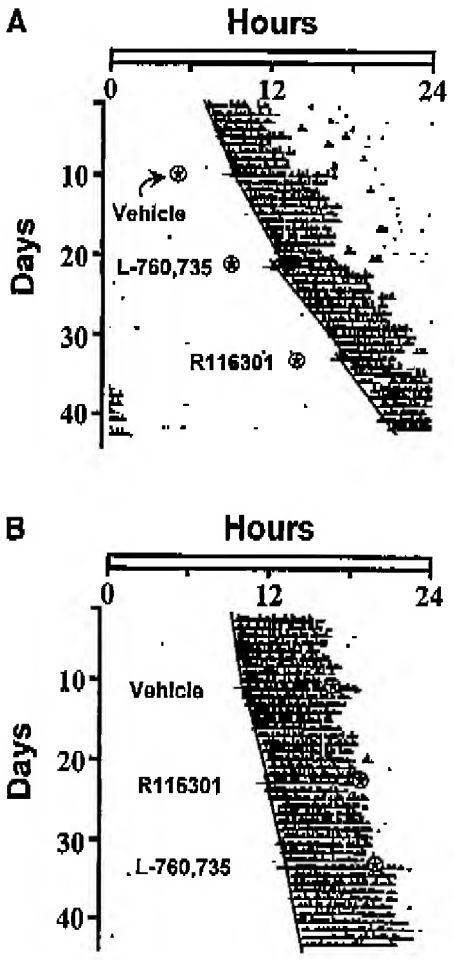

Fig. 5. Wheel-running activity of two hamsters housed in constant light. A, this animal was injected every 10 days at CT8 respectively with vehicle, L-760,735 (5 mg/kg), or R116301 (5 mg/kg). Time of injection is indicated by a star. B, this animal was injected every 10 days at CT19 respectively with vehicle, R116301 (5 mg/kg), or L-760,735 (5 mg/kg). Time of injection is indicated by a star.

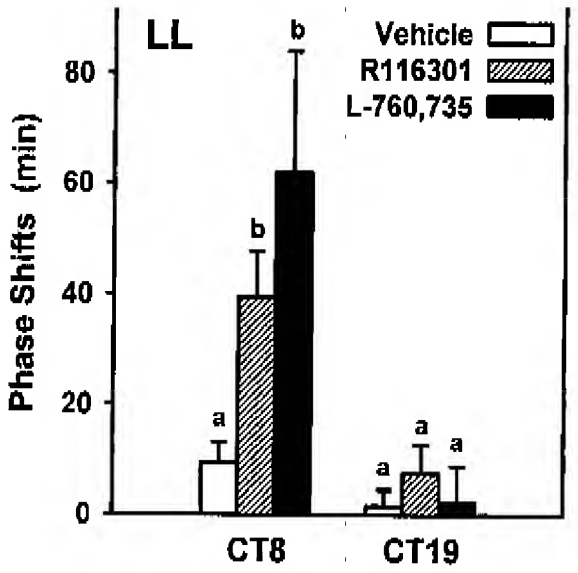

Fig. 6. Phase-advances in circadian activity rhythms of hamsters housed in constant light. Animals were treated with vehicle, R116301 (5 mg/kg) and L-760,735 (5 mg/kg) at CT8 or CT19. Values are means±SEM (n=7 per group). Groups with no letters in common differ significantly from one another (P<0.05).

4. Discussion

This study shows that treatment with a new, selective NK_1 receptor antagonist, R116301, can reduce light-induced phase-advances, while not altering light-induced phase-delays in the circadian rhythm of locomotor activity. Similar effects were found after treatment with L-760,735, another NK_1 receptor antagonist, therefore confirming previous findings obtained with L-760,735 (Challet et al., 1998). Moreover, the present results indicate that modulation of the light-induced phase-advances by the NK_1 antagonists R116301 and L-760,735 is no longer detectable when these antagonists are injected after the light pulse. Because the induction of genes involved in the molecular regulation of photic phase resetting occurs very rapidly (<20 min; e.g., Ginty et al., 1993; Shigeyoshi et al., 1997), these findings suggest that substance P modulates transmission of photic information before it reaches the core of the SCN circadian clock (i.e., at the retina and/or the direct or indirect retino-hypothalamic terminals).

The altered photic phase resetting during the late subjective night but not during the early subjective night raises several possibilities with regard to the site of action of the NK_1 antagonists. First, although the photic sensitivity to irradiance in untreated hamsters remains

constant over the circadian cycle (Nelson and Takahashi, 1991), an inhibition of the retinal NK_1 receptors may have led to a temporary decrease in sensitivity to light in the retina. In this context, a circadian oscillator located in the retina (Tosini and Menaker, 1996) should be mentioned because it may gate the photic cues, possibly with a phase specificity, before these cues reach the SCN clock. An alternative explanation is that, independently of the retina, transmission and/or integration of photic signals in the retino-hypothalamic axons was temporarily impaired. This tachykininergic modulation might have occurred at pre- or post-synaptic levels.

Glutamate is a primary candidate as a neurotransmitter within the retino-hypothalamic tract (Ebling, 1996). Both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors are probably involved in the photic transmission from the retino-hypothalamic tract to the SCN because NMDA and non-NMDA agonists induce phase shifts in vitro with a phase-dependence similar to that of light-induced phase-shifts in behavioral rhythms (Shibata et al., 1994; Ebling, 1996). In vitro interactions between glutamate and substance P are consistent with the assumption that substance P is colocalized with glutamate in the retino-hypothalamic axons (Shirakawa and Moore, 1994; Hamada et al., 1999). Local injections of MK-801, a non-competitive NMDA antagonist, block both light-induced phase-delays and advances (Colwell and Menaker, 1992; Rea et al., 1993). Interestingly, pre-treatment with a competitive non-NMDA (AMPA) antagonist reduces phase-advances, but not phase-delays (Rea et al., 1993; but see also Colwell and Menaker, 1992), as shown here for pre-treatment with the NK_1 the early subjective night) may reflect common mechanisms of AMPA and NK_1 activity in the photic entrainment pathway to the circadian clock.

At least two neural pathways have been recognized to modulate transmission of photic cues to the circadian clock located in the SCN: one projection from the intergeniculate leaflet releases neuropeptide Y (NPY) into the SCN, while the other projection from the midbrain raphe nuclei contains serotonin. Within the SCN, distribution of NPYergic and serotonergic terminals overlap with the recipient area of the retino-hypothalamic axons, suggesting complex neurochemical interactions at that level (Morin, 1994; Miller et al., 1996). Because both light-induced phase delays and advances are enhanced after injections of NAN-190, a serotonergic antagonist acting on 5-HT_{1A} and possibly 5-HT_7 receptors (Rea et al., 1995), serotonin might not interfere with the effects of the NK_1 antagonists that reduce only the photic phase advances. In contrast, local injections of NPY block light-induced phase advances of circadian rhythms, but do not affect light-induced phase delays (Weber and Rea, 1997), that is, with a phase specificity similar to that of the NK_1 antagonists (this study). Therefore, it is possible that treatment with NK_1 antagonists leads to an increase in NPY activity at the level of retino-hypothalamic terminals. Given the multitude of possible interactions between the various inputs to the SCN, it is too early to define where the NK_1 antagonists act to modulate transmission of photic signals upstream of phase resetting mechanisms that occur in the central core of the circadian clock.

The present study also shows that treatment with the NK_1 antagonists R116301 or L-760,735 induces phase-advances in the locomotor activity rhythm in hamsters kept in constant light. These phase-advances were observed when the drugs were administered during the middle of the subjective day (CT8), but no phase-shift was detectable after injections during the late subjective night (CT19). We have previously shown that no phase-shift occurs when L-760,735 is injected during the mid-subjective day to hamsters housed in constant darkness (Challet et al., 1998). Because immunoreactivity of substance P in the SCN increases after two weeks in constant bright light (Takatsuji and Tohyama, 1993), the light background used here (100 lux) may have increased the sensitivity of the circadian system to the NK_1 receptor antagonists.

Given that dark pulses applied at CT8 and CT19 in constant light lead typically to phase-advances and delays in the circadian rhythm of locomotor activity, respectively (Boulos and Rusak, 1982; Ellis et al., 1982; Van Reeth and Turek, 1989), our data show that the effects of the NK_1 antagonists do not match exactly those of dark pulses, suggesting distinct mechanisms at by the dark pulses is critical for the occurrence of the dark-induced phase-shifts, indicating that dark pulses affect circadian rhythmicity through a behavioral activation rather than by modulation of photic inputs (Reebs et al., 1989; Van Reeth and Turek, 1989). In contrast, injections of the NK_1 antagonist L-760,735 in constant light do not trigger locomotor activity (Challet et al., 1998; see also Fig. 5). Furthermore, contrary to the effects of classical non-photic stimuli including behavioral activation (e.g., Mrosovsky et al., 1989), treatment with a NK_1 receptor antagonist has no phase-shifting effect during the mid-subjective day in hamsters maintained in constant darkness (Challet et al., 1998). Taken together, these findings suggest that an inhibition of NK_1 receptor activity alters photic inputs, possibly by signaling "darkness" to the SCN clock.

The NK_1 antagonists produce phase-advances in the free-running rhythm of locomotor activity of hamsters housed in constant light only if the drugs are applied during the subjective day, a time when phase-advances of the SCN clock in vitro can be induced by activation of a cAMP-dependent signaling pathway (Prosser and

Gillette, 1989). Pituitary adenylate cyclase-activating peptide (PACAP), which is localized in the retino-hypothalamic terminals, may be involved in this cAMP pathway (Hannibal et al., 1997) as well as in a calcium signaling pathway (Kopp et al., 1999). Doses of PACAP greater than 10 nM induce phase-advances in the SCN clock in vitro only during the middle of the subjective day (Hannibal et al., 1997; Harrington et al., 1999), as do the NK_1 antagonists injected in hamsters kept in constant light. Many PACAP-sensitive neurons of the SCN are also responsive to substance P (and/or glutamate; Kopp et al., 1999). Thus, PACAPergic and tachykinergic activity may interact in retino-hypothalamic terminals during daytime to modulate phase resetting of the SCN clock.

Depressive illness has been associated with abnormal timing in the circadian clock system (Rosenwasser and Wirz-Justice, 1997). Human aging also is associated with a disruption of circadian rhythmicity. In addition, the circadian clock of many healthy people must be reset following rapid travel across time zones or to accommodate their working schedules. Thus, there is a great need to develop new drugs that could influence the human circadian clock and the circadian control of the sleep–wake cycle. The first step on the development of drugs to alter the human circadian clock is to characterize drugs that are capable of altering the circadian clock of animals. The human circadian clock shares many properties with clocks in lower mammals and it appears that the physiological basis of circadian rhythmicity has been highly conserved throughout mammalian evolution. Given the present results and the prominence of NK_1 receptor expression within the circadian timing system, it is very likely that the NK_1 antagonists R116301 and L-760,735 injected i.p. are acting directly on the circadian clock system, probably on the light input pathway to the clock. The results presented here indicate that NK_1 antagonists may be useful as chronobiological agents in humans.

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